Original Article

MiRNA 3613-5p and MiRNA 3916 rescued the inhibition of cell migration in CNOT2 depleted MDA-MD-231 cells

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Background: The CCR4-NOT complex (CNOT) plays an important role in regulating translation repression. Here, silencing of the complex via the transfection of MDA-MB-231 breast cancer cells with CNOT2 Small interfering RNA (siRNA) decreased the mRNA expression of DiGeorge Syndrome Critical Region 8 (DGCR8) and Dicer.

Methods: Gene expression profiling using an miRNA array was carried out with CNOT2 siRNA treated MDA-MB-231 cells. After transfection with CNOT2 siRNA, qRT-PCR was used to see the level of Dicer and DGCR8. PANTHER pathway analysis was used to see the biological function of microRNAs (miRNAs or miRs).

Results: CNOT2 siRNAs were attenuated the mRNA levels of Dicer and DGCR8. An analysis of miRNAs in CNOT2 silenced MDA-MB-231 cells using miRNA array revealed that 42 miRNAs, including has-miR-7, has-miR-4283, has-miR-10a were significantly upregulated while 47 miRNAs, including has-miR-3916 and has-miR-3613-5p were downregulated following CNOT2 silencing in MDA-MB-231 cells. Also, has-miR-3613-5p and has-miR-3916 rescued the inhibition of migration from CNOT2 short hairpin RNA (shRNA) MDA-MB-231 stable cell lines. PANTHER pathway analysis assigned the miRNAs to multiple processes, including Wnt signaling, angiogenesis, cadherin signaling, inflammation mediated by chemokine and cytokine signaling, integrin signaling, EGF receptor signaling and Huntington's disease.

Conclusions: Together, our findings provide useful target genes for understanding the molecular mechanisms of CNOT2 in breast cancer.

Keywords: CCR4-NOT complex (CNOT); Dicer; Drosha; microRNA; MDA-MB-231cells

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Introduction

The CCR4-NOT complex (CNOT), which is composed of nine core subunits (CNOT1, CNOT2, CNOT3, CNOT6, CNOT6L, CNOT7, CNOT8, CNOT9/RQCD1, and CNOT10), is a large (>2 MDa) multisubunit, multifunctional regulator (1) with multiple cellular functions, including effects on mRNA stability (2), translation (3), and transcription mediated by RNA polymerases I and II. Furthermore, CNOT functions in transcription by repressing TATA-less core promoters. In yeast, CNOT regulates gene expression and controls both cell growth and glucose metabolism (4).

Human CNOT2 is important for maintaining the deadenylase activity and structural integrity of CNOT. CNOT2 depletion was shown to induce apoptosis in a caspase-dependent manner in HeLa cells (5), and was found to interact with cyclin-dependent kinase 11, which is

Table 1 Primers for real-time quantitative RT-PCR (RT-qPCR)

Gene	Forward primers (5' to 3')	Reverse primers (5' to 3')
CNOT2	GGTAACCCAACTCCATTAATAAACC	TGCTGGTTTTGTTACCATTCC
DGCR8	GCAAGATGCACCCACAAAGA	TTGAGGACACGCTGCATGTAC
Dicer	TTAACCTTTTGGTGTTTGATGAGTGT	GGACATGATGGACAATTTTCACA
GAPDH	CCA CTC CTC CAC CTT TGA C	ACC CTG TTG CTG TAG CCA

cleaved by caspases (6). CNOT2 also plays multiple roles in autophagy, adipogenesis, angiogenesis, cell division, bone formation, cell proliferation, and senescence (5,7-10), and CNOT represses promoter activity (11). Furthermore, CNOT modulates the deadenylation of microRNA (miRNA or miR) and Piwi-interacting RNA-targeted mRNAs (12,13). Finally, CNOT interacts with Trinucleotide Repeat Containing 6 (TNRC6 or GW182) which regulates miRNA repression complexes (14,15).

miRNAs are small, noncoding RNAs that regulate gene expression at the post-transcriptional level by repressing mRNA translation or degradation (16). miRNAs have multiple biological functions, including cell growth, apoptosis, differentiation, and proliferation (17). In this study, we found that CNOT2 modulates mRNA level of Dicer, and DiGeorge Syndrome Critical Region 8 (DGCR8) activity and regulates miRNA expression in MDA-MB-231 cells. Also, has-miR-3613-5p and has-miR-3916 rescued the inhibition of migration from CNOT2 depleted MDA-MB-231 cell lines.

We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/tcr-19-2821).

Methods

Cell culture

Human breast cancer cells (MDA-MB-231, RRID: CVCL_0062) were obtained from the American Type Culture Collection (Bethesda, MD, USA) and cultured in RPMI1640 medium (Welgene, Daegu, South Korea) with 2 μM L-glutamine, 10% fetal bovine serum, and penicillin/streptomycin (WELGENE, Korea) under 5% CO_2.

Real-time quantitative RT-PCR (RT-qPCR)

Total RNA from MDA-MB-231 cells was extracted using QIAzol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA using a Reverse Transcription Kit (Promega, Madison, WI, USA). A quantitative analysis was performed with SYBRgreen master mix and carried out using a LightCycler™ instrument (Roche Applied Sciences, Indianapolis, IN, USA). The primers were as listed in *Table 1*.

RNA interference

Control or CNOT2 small interfering RNA (siRNA #1. Cat 108569, siRNA#108571; Thermo Fisher Scientific, Waltham, MA, USA) was transfected into MDA-MB-231 cells using Lipofectamine Transfection Reagent (Invitrogen, USA) according to the manufacturer's protocol.

Generation of CNOT2 short hairpin RNA (shRNA) stably transfected cells

To generate MDA-MB-231 stable cell line for depletion of CNOT2 shRNA (TRCN0000015129CCGGCGG GTTACTAACATTCCTCAACTCGAGTTGAGGA ATGTTAGTAACCCGTTTTT), control (shControl; pLKO.1puro; SHC002, Sigma-Aldrich) were transfected into MDA-MD-231 cells using the Lipofetamine reagent (Thermo scientific, USA) according to the manufacturer's protocol.

miRNA array

miRNA expression profiling was carried out using total RNA from MDA-MB-231 cells treated with CNOT2 siRNA as described previously (18). For miRNA profiling, Affymetrix GeneChip® miRNA 4.0 (Thermo Fisher Scientific Inc., USA) was used. Total RNA was extracted using QIAzol (Invitrogen, USA) according to the manufacturer's protocol.

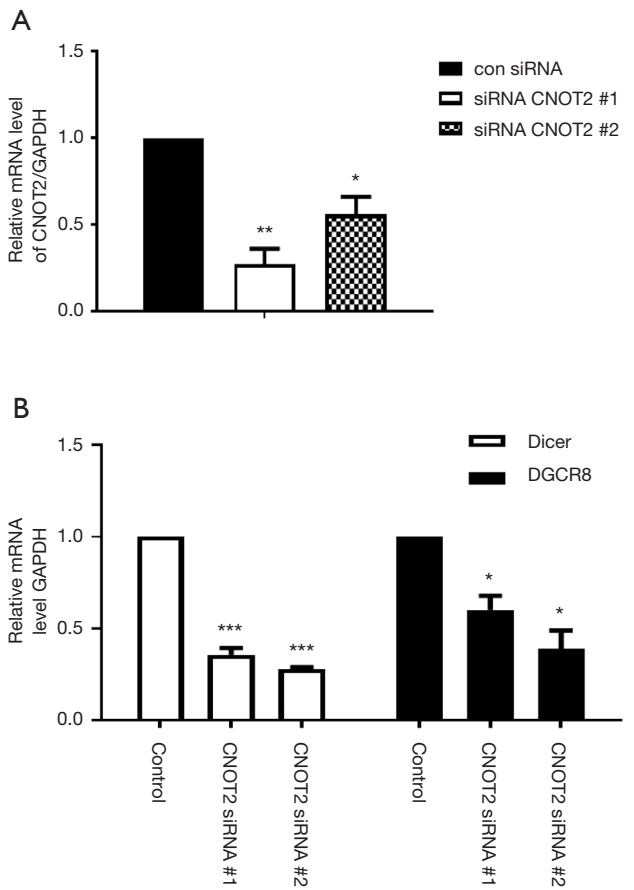

Figure 1 The silencing of CCR4-NOT complex 2 (CNOT 2) decreased the mRNA expression of DGCR8 and Dicer. (A) The small interfering RNA (siRNA)-mediated knockdown of CNOT2 in MDA-MB-231 cells attenuating CNOT2; (B) the expression levels of DiGeorge Syndrome Critical Region 8 (DGCR8) and Dicer. Following transfection of CNOT2 siRNAs, total RNA was collected and real-time quantitative RT-PCR (RT-qPCR) was performed. The data are presented as the mean ± standard error of the mean (SEM) of triplicate samples (n=3). *, P<0.05; **, P<0.01; ***, P<0.001.

Wound healing assay

miRNA mimics (Genolution, Korea) were transfected using INTERFER in siRNA transfection reagent (Polyplus-transfection, USA). After 48 transfection, a wound was generated using a sterile plastic pipette tip and were incubated for 24 h. The cells were fixed and stained with 1% crystal violet (Sigma-Aldrich, USA). To visualize the wound area, an inverted microscope was used.

Pathway analysis

To see the function of differentially expressed miRNAs, target of miRNAs were used for the Protein Analysis Through Evolutionary Relationship (PANTHER) classification system and analysis tools.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The data were analyzed using *one-way* analysis of variance (*one-way ANOVA*) with SigmaPlot (SYSTAT Software Inc., Chicago, IL, USA).

Results

CNOT2 regulates Dicer, and DGCR8

To determine whether CNOT2 affects miRNA biogenesis, we examined the mRNA levels of the miRNA processors Dicer, and DGCR8 using MDA-MB-231 cells treated with CNOT2 siRNA. In MDA-MB-231 breast cancer cells in which CNOT2 was knocked down by two siRNAs targeting different regions of the protein (*Figure 1A*), while the mRNA levels of Dicer and DGCR8 were attenuated (*Figure 1B*).

MiRNA expression following CNOT2 silencing

To determine whether CNOT2 regulates miRNA expression, we conducted gene expression profiling using an miRNA array and CNOT2 siRNA-treated MDA-MB-231 cells. The siRNA-induced silencing of CNOT2 upregulated the expression of 42 miRNAs, including has-miR-7, has-miR-4283, has-miR-10a, and has-miR-200c, whereas 47 genes, including has-miR-3916 and has-miR-3613-5p, were downregulated (*Figure S1*). The top 10 up- or down-expressed miRNAs from CNOT2 siRNA treated MDA-MB-23 cells are listed in *Figure 2A*. We also confirmed that has-miR-7, has-miR-4283, has-miR-200c, and has-miR-10a were upregulated in CNOT2 siRNA-treated cells (*Figure 2B*). The gene ontology annotations, according to the PANTHER PATHWAY database, included ten biological processes, including angiogenesis, Wnt signaling, and Huntington's disease (*Figure 3*).

A

Up-regulated microRNAs (miRs)	Fold change
has-miR-4283	5.385
has-miR-1301	3.675
has-miR-4445-3p	3.381
has-miR-200c	3.338
has-miR-4535	3.271
has-miR-1229	3.079
has-miR-548z	2.978
has-miR-4455	2.846
has-miR-4731-3p	2.745
has-miR-570	2.745

Down-regulated microRNA(miRs)	Fold change
hsa-miR-3613-5p	0.202
has-miR-3916	0.253
has-miR-939	0.268
has-miR-4269	0.325
has-miR-4426	0.372
has-miR-4776-5p	0.336
has-miR-3934	0.382
has-miR-3937	0.391
has-miR-377	0.402
has-miR-3185	0.406

B

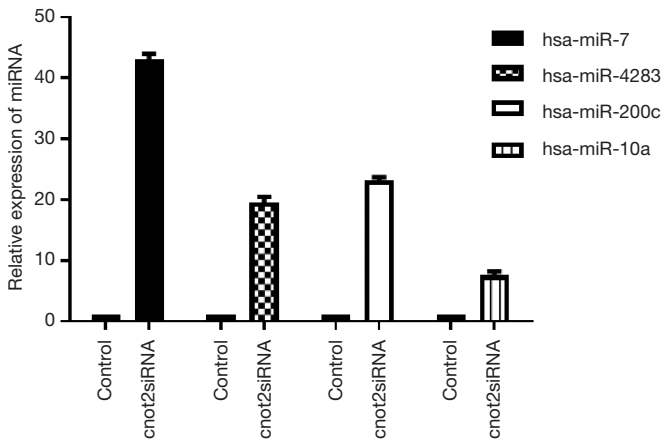

Figure 2 Altered microRNA (miRNA or miR) expression due to the silencing of CNOT2 in MDA-MB-231 cells. (A) List of the 10 most up- or down-regulated miRNAs from CNOT2 siRNA-treated MDA-MB-231 cells. (B) Has-miR-7, has-miR-4283, has-miR-200c, and has-miR-10a were highly expressed in CNOT2 siRNA-treated MDA-MD-231 cells. Following CNOT2 siRNA transfection, total RNA was collected and RT-qPCR was performed. The data are presented as the mean ± SEM of triplicate samples (n=3).

has-miR- 3613-5p and has-miR-3916 mimics rescued the inhibition of cell migration in CNOT2 depleted MDA-MD-231 cells

It was reported that silence of CNOT2 blocked migration of MDA-MB-231 cells (19). To see whether the miRNAs from miRNA array affect the migration, we performed the migration assay. We used has-miR-3613-5p and has-miR-3916 mimics which were the most downregulated from the miRNA array from CNOT2 siRNA treated MDA-MD-231 cells. The result showed that has-miR-3613-5p and has-miR-3916 rescued the inhibition of migration from CNOT2 shRNA stable cell lines (*Figure 4*).

Discussion

In the present study, a CNOT2 deficiency in MDA-MB-231 cells was found to modulate the expression of Dicer, and DGCR8, which are essential for miRNA processing. In addition, we examined miRNA expression in CNOT2 silenced MDA-MB231 cells. Overexpression by has-miR-3613-5p or has-miR-3916 mimic rescued the inhibition

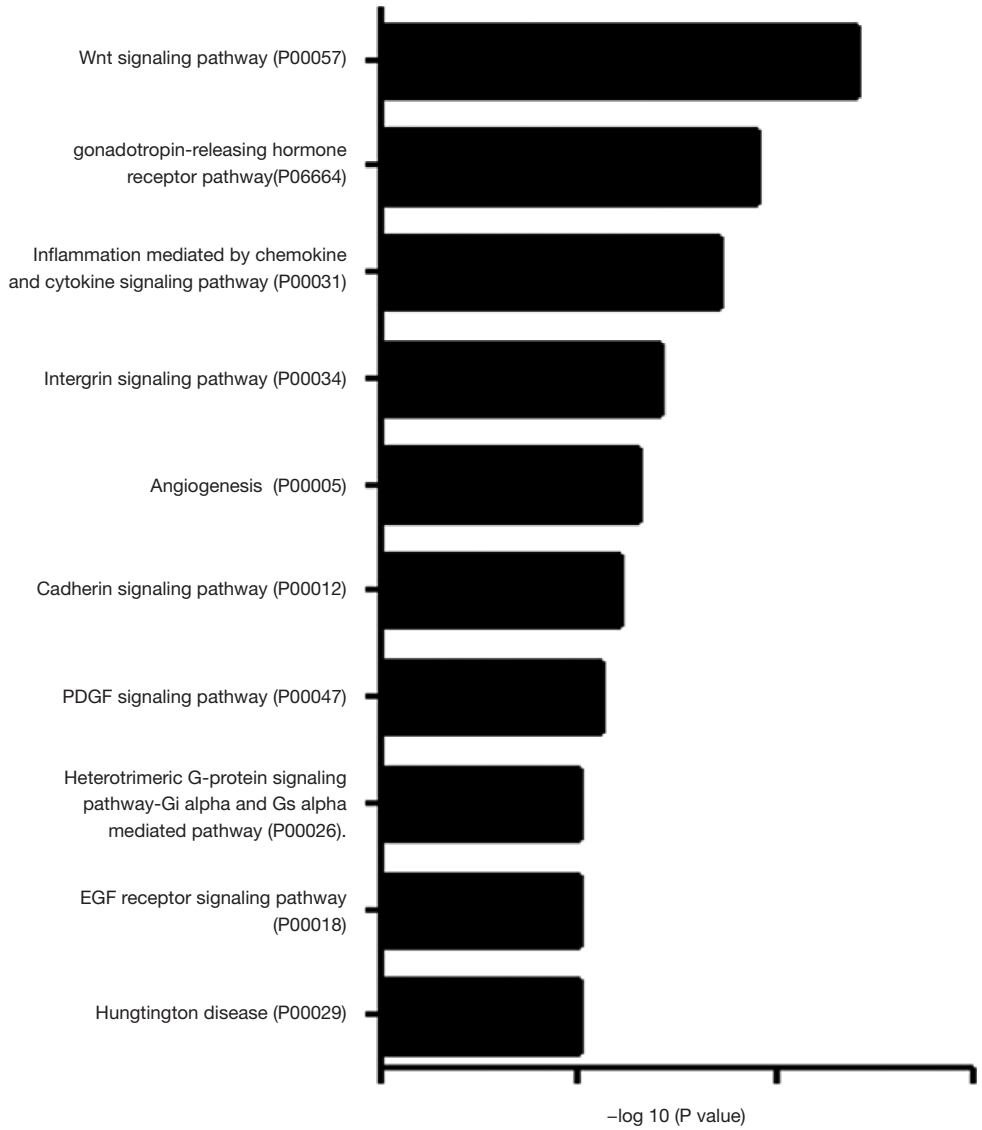

Figure 3 PANTHER (Protein Analysis Through Evolutionary Relationships) for the potential targets of miRNAs.

of migration from CNOT2 shRNA stable cell lines. Furthermore, we analyzed the pathways corresponding to the miRNA targeted genes to determine the function of CNOT2.

There is evidence that CCR4-NOT complex plays an important role in miRNA regulation (13). CNOT1 by interacting with MIF 4G- DEAD-box protein (DDX)6 contributes the repression of miRNA regulation (13). Also, GW182 repressed the function of miRNA by recruiting CNOT Complex (3). Here, we showed that CNOT2 regulates the mRNA level of Dicer, and DGCR8 which are miRNA processing complex and modulates miRNA expression in MDA-MB-231 cells.

It was reported that CNOT2 enhanced proliferation of MDA-MB-231 cells (19). Our study showed that has-miR-3613-5p and has-miR-3916 overexpression which were downregulated in miRNA array of CNOT2 depleted MDA-MB231 cells, rescued the inhibition of migration from CNOT2 shRNA stable cell lines. Thus, our data imply that has-miR- 3613-5p and has-miR-3916 might be important key factors in proliferation of CNOT2 in MDA-MB-231 cells.

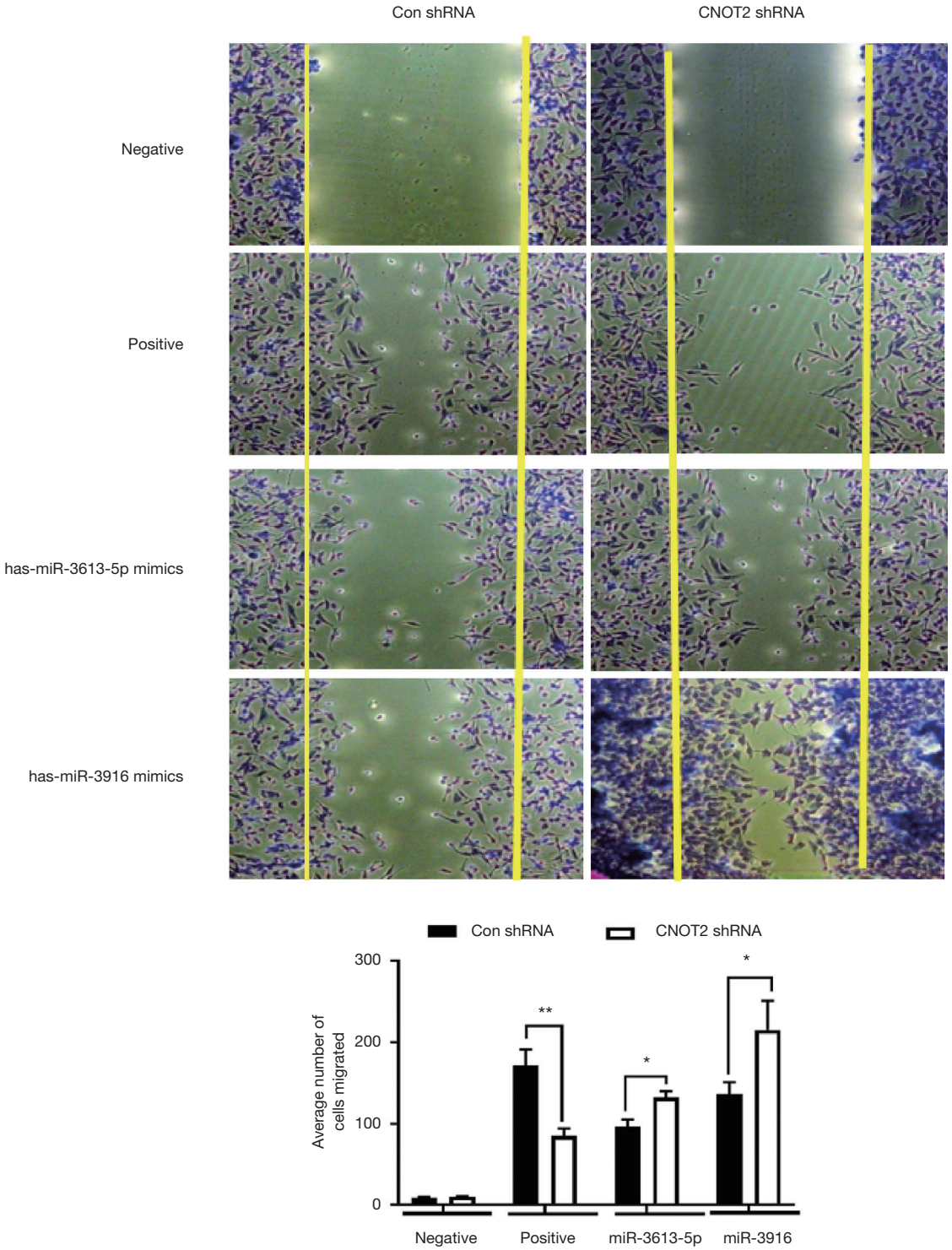

Figure 4 Has-miR-3613-5p and has-miR-3916 mimics rescued the inhibition of cell migration in CNOT2 depleted MDA-MD-231 cells. Has-miR-3613-5p and has-miR-3916 rescued the inhibition of migration from CNOT2 short hairpin RNA (shRNA) stable cell lines by wound healing assay. After 48 h transfection with miRNA mimic, a wound was generated with a plastic tip in control or CNOT2 shRNA stably transfected cells. Negative and positive control indicate scratch before and after cell migration. Data are presented as the mean ± SEM of triplicate samples. *, P<0.05; **, P<0.01.

Our analysis of miRNA within the PANTHER pathway database of miRNA targeted genes from CNOT2 depleted MDA-MB231 cells showed the following ontology: Huntington's disease, Gi alpha and Gs alpha mediated pathway , and platelet-derived growth factor (PDGF) signaling pathway. Our data indicate that has-miR27-awas downregulated in CNOT2 siRNA-treated MDA-MB-231 cells. Chandrasekaran *et al.* (20) showed that has-miR27-a is a potential target in Huntington's disease. has-miR-200c was upregulated while has-miR-21 was downregulated in the array produced from CNOT2 siRNA treated MDA-MB-231 cells, have been studied in relation to the PDGF signaling pathway. For instance, PDGF-BB treatment induced has-miR-200c in vascular smooth muscle cells (21). Furthermore, Wei *et al.* (22) showed that the inhibition of has-miR-21 expression by anti-has-miR-21 antibodies in LX-2 human hepatic stellate cells blocked PDGF-BB-stimulated LX-2 cell activation. The target genes of the miRNA in CNOT2 siRNA treated cells included SP-1 and BCAE-1, which are essential in the pathogenesis of neuroinflammation-associated diseases such as Alzheimer's or Huntington's disease (22).

In conclusion, the current study provides useful target genes and pathways for understanding the molecular mechanisms of CNOT2.

Acknowledgments

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Footnote

Reporting Checklist: The author has completed the MDAR checklist. Available at http://dx.doi.org/10.21037/tcr-19-2821

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tcr-19-2821

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Conflicts of Interest: The author has completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-19-2821). The author has no conflicts of interest to declare.

Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Supplementary

Supplementary. Up-regulated and down-regulated microRNAs (miRs or miRNAs) by CNOT2 siRNA in MDA-MB-231cells. Red indicates upregulated miRNAs, blue indicates downregulation miRNAs. Fold change| ≥ 2 and p value < 0.05

miRNAs	Fold
hsa-miR-4283_st	5.383
hsa-miR-1301_st	3.671
hsa-miR-4445-3p-st	3.331
hsa-miR-200c_st	3.338
hsa-miR-4535_st	3.271
hsa-miR-1229_st	3.079
hsa-miR-548z_st	2.978
hsa-miR-4455_st	2.846
hsa-miR-4731-3p_st	2.745
hsa-miR-570_st	2.745
hsa-miR-139-3p_st	2.672
hsa-miR-769-5p_st	2.663
hsa-miR-3687_st	2.580
hsa-miR-16-2-3p-st	2.557
hsa-miR-1273e_st	2.549
hsa-miR-1271_st	2.434
hsa-miR-3616-3p_st	2.488
hsa-miR-3910_st	2.339
hsa-miR-2110_st	2.339
hsa-miR-3927_st	2.380
hsa-miR-4763-5p_st	2.380
hsa-miR-7_st	2.380
hsa-miR-1287_st	2.378
hsa-miR-769-3p_st	2.300
hsa-miR-548q_st	2.248
hsa-miR-488_st	2.185
hsa-miR-4263_st	2.185
hsa-miR-4770_st	2.173
hsa-miR-520e_st	2.173
hsa-miR-3689a-5p_st	2.178
hsa-miR-548a-3p_st	2.159
hsa-miR-3942-5p_st	2.141
hsa-miR-3163_st	2.209
hsa-miR-3120-5p_st	2.157
hsa-miR-664a-5p-st	2.157
hsa-miR-10a-3p-st	2.054
has - let-7i-3p_st	2.097
hsa-miR-548x_st	2.097
hsa-miR-526a_st	2.048
hsa-miR-623_st	2.048
hsa-miR-486-3p_st	2.002
has- 518c-5p_st	2.002

miRNAs	Fold
hsa-miR-3613-5p_st	0.202
hsa-miR-3916_st	0.253
hsa-miR-939_st	0.268
hsa-miR-4269_st	0.325
hsa-miR-4426_st	0.372
hsa-miR-4776-5p_st	0.336
hsa-miR-3934_st	0.382
hsa-miR-3937_st	0.391
hsa-miR-377_st	0.402
hsa-miR-3185_st	0.406
hsa-miR-92a-5p-st	0.407
hsa-miR-3180-3p_st	0.407
hsa-miR-4513_st	0.409
hsa-miR-4327_st	0.409
hsa-miR-135a-3p-st	0.410
hsa-miR-4486_st	0.419
hsa-miR-150-3p-st	0.421
hsa-miR-1285_st	0.428
hsa-miR-4639-3p_st	0.434
hsa-miR-181-3p-st	0.434
hsa-miR-3942-3p_st	0.439
hsa-miR-3942-3p_st	0.439
hsa-miR-4725-3p_st	0.441
hsa-miR-25-5p-st	0.441
hsa-miR-1260_st	0.441
hsa-miR-665_st	0.446
hsa-miR-1909-5p-st	0.454
hsa-miR-4706_st	0.458
hsa-miR-4450_st	0.458
hsa-miR-1208_st	0.458
hsa-miR-374a_st	0.464
hsa-miR-3126-5p_st	0.468
hsa-miR-4498_st	0.465
hsa-miR-1909_st	0.472
hsa-miR-483-5p_st	0.473
hsa-miR-3064-5p_st	0.477
hsa-miR-4722-5p_st	0.480
hsa-miR-4539_st	0.484
hsa-miR-4707-5p_st	0.495
hsa-miR-27b-5p-st	0.496
hsa-miR-4499_st	0.472
hsa-miR-4491_st	0.477
hsa-miR-1184_st	0.480
hsa-miR-27a-5p-st	0.487
hsa-miR-4282_st	0.487
hsa-miR-21-3p-st	0.498
hsa-miR-4440_st	0.500

Figure S1 Up-regulated and down-regulated microRNAs (miRs or miRNAs) by CNOT2 siRNA in MDM-MB-231cells. Red indicates upregulated miRNAs, blue indicates downregulation miRNAs. |Fold change| ≥2 and P value <0.05.